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The anticonvulsant action of the galanin receptor agonist NAX-5055 involves modulation of both excitatory- and inhibitory neurotransmission

Anne B. Walls[a,*], Sean P. Flynn[b], Peter J. West[b], Margit S. Müller[a,c], Lasse K. Bak[a], Grzegorz Bulaj[d], Arne Schousboe[a], H. Steve White[b]

[a] Department of Drug Design and Pharmacology, Faculty of Health and Medical Sciences, University of Copenhagen, Universitetsparken 2, Copenhagen DK-2100, Denmark
[b] Anticonvulsant Drug Development Program, Department of Pharmacology and Toxicology, College of Pharmacy, University of Utah, 417 Wakara Way, Suite 3211, Salt Lake City, UT 84108, USA
[c] Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1PD, UK
[d] University of Utah, Department Medicinal Chemistry, College of Pharmacy, 30 S 2000 E RM 201, Salt Lake City, UT 84112, USA

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ABSTRACT

The endogenous neuropeptide galanin is ubiquitously expressed throughout the mammalian brain. Through the galanin receptors GalR1-3, galanin has been demonstrated to modulate both glutamatergic and GABAergic neurotransmission, and this appears to be important in epilepsy and seizure activity. Accordingly, galanin analogues are likely to provide a new approach to seizure management. However, since peptides are generally poor candidates for therapeutic agents due to their poor metabolic stability and low brain bioavailability, a search for alternative strategies for the development of galanin-based anticonvulsant drugs was prompted. Based on this, a rationally designed GalR1 preferring galanin analogue, NAX-5055, was synthesized. This compound demonstrates anti-convulsant actions in several animal models of epilepsy. However, the alterations at the cellular level leading to this anti-convulsant action of NAX-5055 are not known. Here we investigate the action of NAX-5055 at the cellular level by determining its effects on excitatory and inhibitory neurotransmission, *i.e.* vesicular release of glutamate and GABA, respectively, in cerebellar, neocortical and hippocampal preparations. In addition, its effects on cell viability and neurotransmitter transporter capacity were examined to evaluate potential cell toxicity mediated by NAX-5055. It was found that vesicular release of glutamate was reduced concentration-dependently by NAX-5055 in the range from 0.1 to 1000 nM. Moreover, exposure to 1 μM NAX-5055 led to a reduction in the extracellular level of glutamate and an elevation of the extracellular level of GABA. Altogether these findings may at least partly explain the anti-convulsant effect of NAX-5055 observed *in vivo*.

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1. Introduction

The most abundant excitatory and inhibitory neurotransmitters in the mammalian brain are glutamate and γ-aminobutyric acid (GABA), respectively. Under physiological conditions the ratio between glutamatergic and GABAergic synapses is about 9 (Attwell and Laughlin, 2001) and an imbalance in this relationship appears to be involved in a number of neurological disorders such as Alzheimer's disease, schizophrenia and epilepsy (Sonnewald and Kondziella, 2003; Barker-Haliski et al., 2014; Schousboe et al., 2014). Glutamatergic and GABAergic neurotransmission are both mediated through Ca^{2+} dependent vesicular release in the synapse and subsequent interaction with receptors in

Abbreviations: ANOVA, analysis of variance; AUC, area under the curve; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's Modified Essential Medium; f.c., final concentration; fEPSPs, field excitatory post synaptic potentials; GABAγ, -aminobutyric acid; GBSS, Gey's balanced salt solution; GVGγ, -vinyl GABA (vigabatrin); IR-DIC, infrared differential interference contrast; LSD, least significant difference; mEPSC, miniature excitatory post synaptic currents; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NMDA, *N*-methyl-D-aspartate; PBS, phosphate buffered saline; PBT, PBS +0.3% Triton-X; PPF, paired-pulse facilitation; PTX, picrotoxin; TTX, tetrodotoxin; R_a, access resistance; R_m, membrane resistance; SDS, sodium laurylsulfate; SEM, standard error of the mean.

* Corresponding author. Tel.: +45 3533 6363.
E-mail address: abw@sund.ku.dk (A.B. Walls).

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the postsynaptic membrane. The receptor interaction is terminated by clearance of the neurotransmitter from the synaptic space by high affinity transporters located on the pre-synaptic neurons and adjacent astrocytic processes (Schousboe, 2003; Schousboe et al., 2013).

Galanin, an endogenous neuropeptide, is widely distributed in the central nervous system and it exerts its action *via* three different galanin receptors, named GalR1-3. Based on mRNA expression, these three receptors appear to be expressed throughout the brain, although with some regional differences in intensities and relative subtype expression (O'Donnell et al., 1999; Waters and Krause, 2000). Specifically, by binding to GalR1 or GalR2 galanin is able to modulate both glutamatergic and GABAergic transmission (Zini et al., 1993b; Mazarati et al., 2000; McColl et al., 2006) and thus galanin is likely to be a key player in fine-tuning the balance between excitatory and inhibitory transmission required for proper brain function. Employing several different genetic knockout mouse models it was demonstrated that galanin displays anti-convulsant effects involving increased seizure threshold as well as reduced seizure severity and duration (Mazarati et al., 2000; McColl et al., 2006; Mitsukawa et al., 2008). However, while the activation of GalR1 receptors appears to elevate seizure threshold as well as reduce severity and duration of seizures stimulation of GalR2 receptors only seem to affect seizures in the maintenance phase but do not prevent their initiation (Pooga et al., 1998). Accordingly, agonists targeting GalR1 are likely to be a suitable approach in the search for novel therapeutic agents against seizure activity and epileptogenesis.

Taking into account that peptides are generally poor candidates for therapeutic agents due to their low bioavailability, and that galanin does not penetrate the blood-brain barrier, a search for alternative strategies for the development of galanin-based anti-convulsant drugs was prompted. The galanin analogue NAX-5055 is a rationally designed peptide which has good receptor subtype specificity being approximately 15 times more potent at GalR1 receptors compared to GalR2 with affinities that resemble those of the native peptide (K_i values are 3.5 nM and 51.5 nM for GalR1 and GalR2, respectively (Bulaj et al., 2008)). Moreover, NAX-5055 crosses the blood brain barrier and has a good metabolic stability with a half-life of 9.4 h in 25% rat serum at 37 °C (Bulaj et al., 2008). Intraperitoneal administration of NAX-5055 protects against seizures in three different mouse models of epilepsy, *i.e.* the Frings audiogenic seizure-susceptible mouse (ED_{50} 3.2 mg/kg), the mouse corneal kindling model of partial seizures (ED_{50} 0.65 mg/kg) and the 6 Hz model of pharmacoresistant epilepsy (ED_{50} 0.7, 0.8 and 2.9 mg/kg for 22, 32 and 44 mA, respectively) (White et al., 2009).

Although the cellular alterations mediated by NAX-5055 which lead to an anticonvulsant action is thought to resemble that of the native peptide galanin, *i.e.* by reducing glutamatergic transmission (Zini et al., 1993b; Mazarati et al., 2000), this hypothesis remains to be confirmed. In this study we examined the effect of NAX-5055 on vesicular release of glutamate and GABA from primary cultures of cerebellar and cerebral cortical neurons, respectively. In addition, effects of NAX-5055 on glutamate release were evaluated electrophysiologically in hippocampal CA3 pyramidal cells employing organotypic hippocampal slices. Finally, we tested the toxicity of NAX-5055 in cultured neurons by measuring mitochondrial function using an MTT assay (Mosmann, 1983) and by assessing neurotransmitter transporter capacity of the cell membrane. For this latter purpose a concentration of NAX-5055 being at least one order of magnitude higher than the concentration effective in attenuating glutamate release was employed.

2. Materials and methods

2.1. Materials

Seven-day-old NMRI mice and pregnant NMRI mice (15 days gestation) were purchased from Harlan (Venray, Netherlands) while C57BL/6J mice were from Charles River (Kingston, WA, USA). Fetal calf serum was acquired from Seralab Ltd. (Sussex, UK) and culture medium, poly-D-lysine (molecular weight > 300,000 g/mol), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), *N*-methyl-D-aspartate (NMDA), glycine, Gey's balanced salt solution (GBSS), Glutamax, HEPES, Hank's balanced salt solution and horse serum were purchased from Sigma-Aldrich (St. Louis, MO, USA). γ-Vinyl-GABA (GVG; vigabatrin) was obtained from Tocris (Bristol, UK) and D-[^{3}H]aspartate as well as [^{3}H]GABA were purchased from Amersham Biosciences (Buckinghamshire, UK). NAX-5055 used in this investigation was synthesized according to procedures described previously (Bulaj et al., 2008) and was kindly provided by Dr. Grzegorz Bulaj, University of Utah, Salt Lake City and Dr. Brian Klein, NeuroAdjuvants, Inc. (Salt Lake City, UT, USA).

2.2. Organotypic hippocampal slice culture preparation

Organotypic hippocampal slice cultures were prepared using a modification of the method originally described by Stoppini et al. (1991) and Alex et al. (2011). On postnatal day 5 (P5), C57BL/6J mouse pups were anesthetized with pentobarbital (25 mg/ml) and rapidly decapitated. Brains were extracted and the cerebellum was removed. Hemispheres were cut into 350 μm transverse sections using a McIlwain tissue chopper (Stoelting Co., Wood Dale, IL) and placed in chilled GBSS supplemented with 6.5 mg/ml glucose. Sections were separated under a dissection microscope and excess cortex removed to obtain intact hippocampal slices with the entorhinal cortex attached. Hippocampal sections were transferred to tissue culture membrane inserts (Millipore) in a 6-well tissue culture dish containing medium consisting of 50% minimum essential medium with Glutamax and HEPES, 25% Hanks balanced salt solution, 25% heat inactivated horse serum, and 6.5 mg/ml glucose. Medium was changed three times per week. Cultures were maintained at 37 °C with 5% CO_2 balanced humidified air in a standard water-jacketed incubator (Thermo Scientific, Marietta, OH). Organotypic hippocampal slices were used for experiments after 14–21 days *in vitro*.

2.3. Electrophysiological recordings

On the day of recording, a membrane insert with 3–4 hippocampal cultures was removed from the 6-well culture plate and placed in a sterile Petri dish that contained external recording solution containing (in mM): 150NaCl, 3KCl, 2$CaCl_2$, 1.3$MgCl_2$, 10HEPES, 10 glucose at pH 7.30 and osmolarity of 305 mOsm. External solution was stored in the incubator at 37 °C. Slices were removed from Petri dishes by cutting a portion of the membrane insert surrounding a single slice with a sterile scalpel and immediately placed into a perfusion recording chamber. The remaining slices on each insert were returned to the incubator for later use. Once in the recording chamber, slices were submerged and perfused at 2 ml/min with external recording solution. All recordings were performed at room temperature. To isolate miniature excitatory postsynaptic currents (mEPSCs), tetrodotoxin (TTX, 1 μM) and picrotoxin (PTX, 50 μM) were added to the external recording solution immediately before use. Whole cell recording pipettes (3–5 MΩ) were pulled on a PIP5 pipette puller (HEKA Instruments Inc., Bellmore, NY) and filled with

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